AD_____

Award Number: DAMD17-01-1-0028

TITLE: A Genomic Approach to Identifying Novel Targets for Early
Detection and Intervention of Prostate Cancer

PRINCIPAL INVESTIGATOR: Wan L. Lam, Ph.D.
Juergan R. Vielkind

CONTRACTING ORGANIZATION: British Columbia Cancer Agency Research Center
Vancouver, British Columbia V5Z 1L3 Canada

REPORT DATE: September 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

REPORT DOCUMENTATION PAGE

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2002	Annual (20 Aug 01 - 19 Aug 02)

4. TITLE AND SUBTITLE
A Genomic Approach to Identifying Novel Targets for Early Detection and Intervention of Prostate Cancer

5. FUNDING NUMBERS
DAMD17-01-1-0028

6. AUTHOR(S):
Wan L. Lam, Ph.D.
Juergan R. Vielkind

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

British Columbia Cancer Agency Research Center
Vancouver, British Columbia V5Z 1L3 Canada

E-Mail: wanlam@bccancer.bc.ca

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)
Early detection and intervention is key to a favorable prognosis in prostate cancer. Despite advances in the detection and treatment, the mortality rate remains high. To improve survival, early detection and treatment strategies tailored to pre-invasive prostate cancer are required. We propose to catalogue genetic alterations associated with the developmental stages of disease for use as diagnostic tools and to identify the critical genes that drive the transformation of premalignant lesions to tumors for use as molecular targets for novel treatment design. The combination of laser capture microdissection (isolation of specific cell types from hundreds of specimens) and SMAL DNA fingerprinting technology (high-through put analysis of genomic targets using minute quantities of DNA yielded from the microdissected cells) will facilitate systematic comparison of samples in various stages of disease development. By the end of this work, we will have identified a set of genetic loci (and genes) by virtue of their frequency of alteration in premalignant lesions and subsequently in low-grade tumors. We will have established a publicly accessible "genetic alterations in prostate cancer" database which catalogs somatic changes present in the various stages of cancer progression. Such information contribute to the fundamental understanding of prostate cancer pathogenesis.

14. SUBJECT TERMS
prostate cancer, detection, intervention

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Project Title: A Genomic Approach to Identifying Novel Targets For Early Detection and
Intervention of Prostate Cancer

Authors: Wan L. Lam and Juergen R. Vielkind

1. Introduction

1.1. Background

Early detection and intervention is key to a favorable prognosis in prostate cancer (CaP), as in
many types of cancers. Disease progression is thought to be driven by cumulative genetic
alterations affecting a small number of genes (see Kinzler and Vogelstein, 1996). Identification
of these genes would provide novel targets for diagnosis and intervention. Despite many efforts
these genes have not yet been identified. One possibility to discover these genes is by
identifying genetic alterations that parallel the histopathological progression stages in prostate
cancer. This can only be achieved by a genome-wide screen for alterations.

CaP consists of a mixture of normal epithelial cells, stromal cells, benign hyperplastic cells, PIN,
and various clones of invasive carcinoma. To date, due to the difficulty in obtaining sufficient
material from the small early progression stages and the lack of analytical methods applicable for
genome-wide scanning of minute clinical specimens, much of our knowledge on genetic changes
in CaP has been derived from analyses of advanced tumors and cell lines, which are
characterized by complex genetic changes many of which may be random, due to generalized
genetic instability, rather than being etiologic. Thus, genetic alterations relevant to disease
progression may be more easily identified by examining stages earlier in the development of a
tumor, before the accumulation of randomly altered changes occurs. We therefore focus on
identifying genetic changes in premalignant lesions which subsequently appear in the early
invasive stages of CaP. "Normal epithelium" versus "PIN" comparison will reveal the spectrum
of genetic changes in the early stages of CaP development, while the "PIN" to "tumor"
comparison will identify the subset of changes critical to disease progression.

The objective of this proposal is to identify the progressive genetic alterations that cause normal
prostate epithelial cells to transform into precursor prostatic intraepithelial neoplasia and
invasive cancer cells of low Gleason grade. This will be achieved by systematically comparing
high density DNA fingerprints of microdissected samples in various stages of disease
development. Over a 3 year period, we will compare 450 DNA samples extracted from normal
cells, from precancerous cells and from tumor cells in a variety of patient biopsies, in order to
identify key genetic changes in the stages of disease development. Our early results as well as
progress in relation to the original Statement of Work are summarized in this Annual Report.

2. Body

We have proposed to use laser capture microdissection to selectively isolate pure cell
populations representing normal epithelium, benign hyperplasia, high grade PIN and invasive
cells of low Gleason grades 1-3. We will couple this cell isolation approach with SMAL-PCR

DNA fingerprinting technology in order to analyze these various CaP progression stages at thousands of randomly distributed genetic loci for frequent alterations. The <u>specific aims</u> are:

1. To generate SMAL DNA fingerprints from normal epithelium, PIN and invasive carcinomas of early stages.
2. To identify recurring alterations that are present in the early progression stages.
3. To establish an expandable database of recurring changes for each stage of disease development.
4. To assign recurring alterations to specific chromosomal regions.
5. To identify candidate genes in the mapped regions for future mutation scanning.
6. To begin validation of candidate genes.

Aims 1-4 are scheduled to begin within Year 1 of the project extending into Years 2 and 3, while aims 5 and 6 are Year 2-3 activities. A comparing our progress against the original Statement of Work is presented in the Reportable Outcomes section below.

2.1. Experimental design and methodology

The methodologies were described in detail in the original proposal. This is a brief description of the two key technologies used in this project.

Laser Capture Microdissection.

To overcome tissue heterogeneity in the prostate biopsy specimens, each sample is evaluated histopathologically and then subjected to microdissection. Serial 5mm sections are prepared and each placed in the centre of an uncoated glass slide facilitating laser capture of the target cells. Slide 1 to 5 are stained with toludine blue. Slides 1-4 will be kept desiccated until used while slide #5 is be coverslipped and used as the reference slide for pathological evaluation. The remaining slides are kept in reserve for verification of experimental result and/or used if not enough cells can be microdissected from slides 1-4. Under direct microscopic observation, a vial cap, which carries a thermoplastic film on its undersurface, is placed over sections from paraffin-embedded or frozen biopsy material. A laser is then aimed over the desired cells in the sections. Upon activation of the laser, the targeted cells are selectively adhered onto the film and can be removed for further analysis (for review of LCM see: Pappalardo et al., 1998; Simone et al., 1998). DNA is extracted from the captured cells. DNA concentrations are determined by quantitative PCR comparing against a standard curve generated from the amplification of known DNA quantities of genomic DNA.

SMAL-PCR DNA fingerprinting. This technique is designed for systematic high density scanning for alterations in cancer cells. Due to the minute quantities of DNA available in microdissected samples, we have modified the conventional Arbitrarily Primed-PCR (AP-PCR) DNA fingerprinting (Peinado et al., 1992; Ionov et al., 1993) technology for analyzing microdissected cells. We have named the new technique <u>S</u>canning of <u>M</u>icrodissected <u>A</u>rchival <u>L</u>esions, SMAL-PCR. As in AP-PCR, SMAL-PCR utilizes multiples of short, arbitrary primer pairs of 10 nucleotides to simultaneously amplify a large number of targets randomly distributed throughout the genome. The resulting PCR fragments are separated on acrylamide gels allowing the identification of polymorphic genetic alterations between, e.g. normal and cancerous tissue.

This technique has yielded much more genetic information than that provided by the conventional assays for detecting chromosomal alterations. We have shown that highly reproducible DNA fingerprints could be generated from < 2 ng of DNA (300 cell equivalents) extracted from cells of various archived tumor specimens.

2.2. Progress in specific aims

Aim 1: Generate SMAL DNA fingerprints from normal epithelium, PIN and tumors

Procurement of archival tissue:
A large number of paraffin embedded CaP biopsies have been evaluated histopathologically in order to identify specimens containing pre-cancerous and cancerous lesions (cells) suitable for microdissection. Under the supervision of 2 pathologist, areas of normal epithelium, PIN and invasive carcinoma were identified.

LCM, DNA extraction and SMAL fingerprinting:
A total of 72 samples have been microdissected. Figure 1 shows an example. DNA has been extracted from each sample and quantified. SMAL-PCR of these samples using selected primer pairs provided DNA fingerprints for comparison (see Aim 2).

**Before**   **After**   **Captured**

Figure 1. Laser capture microdissection of prostatic intraepithelial neoplasia from stained sections from paraffin-embedded radical prostatectomies.

Aim 2: Identify recurring alterations that are present in PINs and tumors

The criterion for the initial screen is that an alteration has to occur more than once in >10% of cases. Recurring polymorphic fragments will be cloned, sequenced and validated in larger cohorts.

Comparison of DNA fingerprints between normal cells, PIN and/or tumors from individual patients revealed genetic alterations (gain or loss of PCR signal). Comparison amongst a panel of patients revealed recurring genetic alterations. For example, see Figure 2. To date, 24 recurrent changes have been identified. Twenty of these recurring changes have been cloned and sequenced. Localization of these sequences to specific chromosome regions (Aim 5) will be performed in Year 2 of this project as scheduled.

6
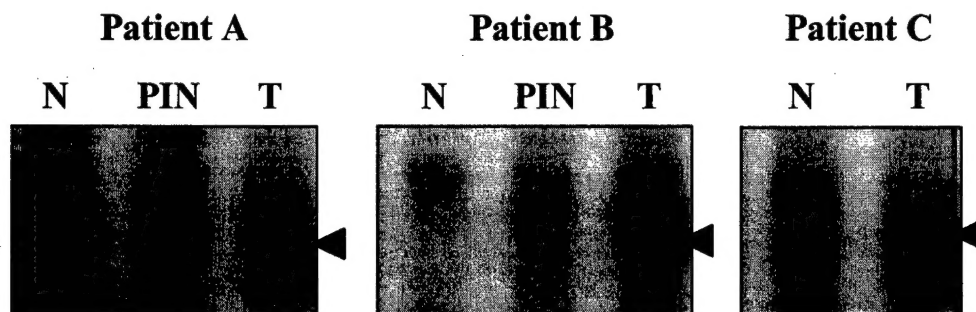
Figure 2. Example of a recurring genetic alteration identified by SMAL-PCR DNA Fingerprint comparison. DNA was extracted from LCM procured cells from formalin-fixed paraffin embedded samples. DNA fingerprint comparison of normal (N), prostatic intraepithelial neoplasia (PIN) and tumor (T) from individual patients revealed a 250bp recurrent gain in tumor sample in 3 out of 14 patients analyzed.

Aim 3: To establish an expandable database of recurring genetic changes

A database of recurring genetic alterations has been established. Information on the 24 recurring changes, such as frequency of occurrence, histopathological stage of sample, sequence of DNA fragment have been entered into this new database.

The database will be updated with chromosomal locations of the alterations as the information appears (see Aim 4). The genes situated within these regions will be added once determined (Aim 5). The depth of this expandable database will grow as increasing numbers of samples are analyzed.

Aim 4: To assign recurring alterations to specific chromosomal regions

The sequence of each SMAL-PCR alteration is being use to identify specific human bacterial artificial chromosome (BAC) clones containing the sequence. The location of the BAC clone in the human genome informs the chromosomal location of the recurring genetic alteration. This effort is underway and will continue in Year 2 and 3.

Aim 5: To identify candidate genes in the mapped regions for future mutation scanning

This aim is scheduled for Year 2 and 3. We have begun candidate genes in the regions of alterations by computational searches of all known cDNA, EST and microarray and SAGE expression data bases.

Aim 6: To begin validation of candidate genes

We anticipate to begin validation of candidate genes (the best two genes) near the end of this proposal.

3. Key Research Accomplishments

The key research accomplishments in Year 1 of this project is listed below:

1. Procurement of a growing panel of histopathologically graded prostate specimens and the identification of 75 lesions suitable for microdissection.

2. Laser capture microdissection of 75 samples. While LCM is simple in principle, each tissue type requires fine tuning of the LCM procedure due to variables in tissue acquisition, fixation and storage. LCM technology has been established in Dr. Vielkind's (co-applicant) laboratory. An Arcturus PixCell II laser capture microdissection device is routinely used to isolate cells or cell clusters from prostate biopsies. Pure cell samples have been isolated from 75 patient specimens. DNA extraction from these samples are completed. This activity will continue in Year 2 and 3.

3. Identification of 24 recurring genetic alterations in PIN and/or prostatic carcinoma. We anticipate that some of these (and future) alterations will map to chromosomal regions previously unknown to be involved in prostate cancer development.

4. Work on laser capture microdissection has been presented at 3 conferences.

> Stephanie Ma, Hans Adomat, Terry Bainbridge, Doug Webber, Wan Lam, Juergen Vielkind A concerted genomic and proteomic approach to understand the early steps in the genesis of prostate cancer. American Association for Cancer Research, San Francisco, CA (April 2002)

> Stephanie Ma, Hans Adomat, Terry Bainbridge, Doug Webber, Wan Lam, Juergen Vielkind. A concerted genomic and proteomic approach to understand the early steps in prostate carcinogenesis. British Columbia Cancer Agency Conference, Vancouver, Canada (Nov 2001)

> Stephanie Ma, Hans Adomat, Terry Bainbridge, Doug Webber, Wan Lam, Juergen Vielkind (2001) A proteomic approach to understand the onset and early progression of prostate cancer using Surface Enhanced Laser Desorption Ionization (SELDI) proteinchip array. Laser Capture Microdissection Symposium, NIH, Washington, DC (July 2001)

5. One Abstract has been published based on our early results.

> Ma S, Adomat H, Bainbridge CT, Webber D, Lam W, Vielkind J (2002) A concerted genomic and proteomiv approach to understanding the early steps in the genesis of prostate cancer. American Assoc. for Cancer Research Proceedings 43: 683.

4. Reportable Outcomes

The scientific accomplishments for Year 1 is listed in section 3 above. This section measures our progress (reportable outcomes) against the original Statement of Work (Task 1-6).

Task 1. To generate DNA fingerprints from normal epithelium, PIN and early invasive carcinomas (months 1-30)
>•case selection and pathology review of 300 cases (months 1-30)
>•laser capture microdissection of 150 samples for each stage (months 1-30)
>•DNA preparation, quantitation and quality control (months 1-30)
>•generate 90,000 DNA fingerprints using 20 primer pairs for each of the 450 samples (months 4-30)
>•collect and process fingerprint images for analysis (months 4-30)

For month 1-12, the following reportable outcomes have been achieved.

- \>100 cases have been reviewed by collaborating pathologists to identify 75 suitable for microdissection
- 72 samples (ie. Normal, PIN, cancer) were microdissected
- DNA was extracted from all 72 samples
- 144 SMAL-PCR profiles were generated using 1 primer pair; on average, 30 fingerprint bands are produced per profile giving an estimate of 4,000 fingerprint bands

Task 2. To identify recurring alterations that are present in the stages (months 4-30)
> • compare pairs of fingerprint images within each histopathological stage to identify recurring alterations (months 4-30)

In this first year, a total of 24 recurrent changes were identified (66 separate bands, originating from the 24 recurrent changes, were isolated). After cloning and sequencing, 20 recurring genetic alterations have been identified to date.

Task 3. To establish an expandable database of recurring changes for each stage of disease development (months 4-30)
- establish and format database
- data entry as information becomes available (months 4-30)
- identify recurring alterations that occur in PIN and are also present in early invasive carcinomas (12-30)

A database of recurrent genetic changes found in pre-cancerous and cancerous specimens has been established. Information such as pathological grades, frequency of occurrence and DNA sequence are included. Chromosomal location of the genetic alterations as well as candidate genes in these regions will be added as they become available (see Aim 3 in Section 2 above)'

Task 4. To assign recurring alterations to specific chromosomal regions (months 12-36)
- clone and sequence selected recurring alterations (months 12-36)
  - Year 1: 10 alterations
  - Year 2: 20 alterations
  - Year 3: 20 alterations
- map sequences to chromosomal locations
  - locate alterations by comparing sequences against available human genome sequence (months 12-36)

9

- locate remaining alterations by hybridization to human BAC library (months 24-36)

Task 4 is due to begin in Year 2. However, we have begun chromosme assignment of the 20 sequenced recurring alterations.

Task 5. To identify candidate genes in the mapped regions for future mutation scanning (months 24-36)
- search cDNA, EST, microarray and SAGE data bases (months 24-36)

Task 5 is schedule for Year 3 and therefore has not been addressed.

Task 6. To begin validation of the best 2 candidate genes. (months 18-36)
- dissection of frozen tissue sections from prostate biopsies
- isolate RNA samples (months 18-36)

mutation detection (months 24-36)
The on-going work has been presented at the following conferences:

Task 6 is schedule for Year 2-3 and therefore has not been addressed.

5. Conclusions

Operationally, the work accomplished in the first year of this project matched that of the proposed Tasks in the Statement of Work and the original proposal. Meeting the milestones suggestes that the required materials, infrastructure and expertise are available and capable of support the work proposed. The following processes have been established: tissue specimen procurement, histopathological evaluation, laser capture microdissection, DNA extraction and quantitation, SMAL-PCR fingerprint comparison, cloning and sequencing, chromosomal localization, databases and bioinformatics.

Scientifically, our early results demonstrate that it is possible to discover recurring genetic alterations in microdissected PINs and invasive carcinoma of the prostate, despite the minute size of such samples.

As mention in the original proposal, by the end of this work, we will have identified a set of genetic loci by virtue of their frequency of alteration in premalignant lesions and subsequently in low grade tumors. We will have established a "genetic alterations in prostate cancer" database which catalogs somatic changes present in the various stages of cancer progression. This database, when completed, will be made publicly and freely accessible on the BC Cancer Research Center web site. Researchers studying cancer progression (prostate and otherwise) will be able to access information and submit their own data. These data will be extremely valuable in identifying targets for early diagnosis and treatment. Future studies will allow cross-referencing of stage-specific fingerprints against clinical outcome and thus will allow relating genotype to disease risk and behavior.

6. References

Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M: Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature **363**: 558-561, 1993.

Kinzler KW, Vogelstein B: Lessons from hereditary colorectal cancer. Cell **87**: 159-170, 1996.

Landis SH, Murray T, Bolden S, Wingo PA: Cancer statistics. CA Cancer J Clin **49**: 8-31, 1999..

Pappalardo PA, Bonner R, Krizman DB, Emmert-Buck MR, Liotta LA: Microdissection, microchip arrays, and molecular analysis of tumor cells (pimary and metastases). Seminars in Radiation Oncology **8**: 217-223, 1998.

Peinado MA, Malkhosyan S, Velazquez A, Perucho M: Isolation and characterization of allelic losses and gains in colorectal tumors by arbitrarily primed polymerase chain reaction. Proc Natl Acad Sci USA **89**: 10065-1069, 1992.

Simone NI, Bonner RF, Gillespie JW, Emmert-Buck MR, Liotta LA: Laser-capture microdissection: opening the microscopic frontier to molecular analysis. Trends Genet **14**: 272-276, 1998.

7. Appendices

None